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14. ABSTRACT The epidermal growth factor receptor (EGFR) is a prototypic receptor tyrosine kinase (RTK) and a member of the ErbB family of receptors. The ErbB receptors and their cognate ligands are frequently overexpressed in human cancers, notably carcinoma of the breast. Unfortunately no known secreted or extracellular ErbB receptor inhibitors have been discovered in mammals. Two natural extracellular inhibitors of the highly homologous <i>Drosophila</i> EGF receptor (dEGFR) have been recently discovered in <i>Drosophila melanogaster</i> . One (Argos) resembles a secreted growth factor, the other (Kekkon) is a transmembrane protein. We have discovered a novel mechanism of EGFR regulation by Argos. Argos inhibits EGFR activation by sequestering activating ligand and preventing the growth factor from binding to and activating the receptor. To get a further structural understanding of this inhibition we have produced crystals of Argos for x-ray crystallographic studies. We are also currently screening crystallization conditions for a complex of Argos bound to an epidermal growth factor (EGF). The structural understanding of this inhibition should help lead to novel strategies to target breast cancer that is ErbB regulated.					
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INTRODUCTION

The epidermal growth factor receptor (EGFR) is a prototypic receptor tyrosine kinase (RTK) and a member of the ErbB family of receptors (1,2). The ErbB receptors and their cognate ligands are frequently overexpressed in human tumors(3), contributing to inappropriate activation of cell growth and promoting transformation of cells(4-7). No secreted or extracellular ErbB receptor inhibitors have been reported in mammals. However, two natural extracellular inhibitors of the highly homologous *Drosophila* EGF receptor (dEGFR) (8,9) are found in *Drosophila melanogaster*(10,11). One (Argos) resembles a secreted growth factor(12), the other (Kekkon) is a membrane bound protein(13). My research proposal therefore focuses on obtaining a detailed mechanistic and structural understanding of how Argos and Kekkon function to antagonize dEGFR activity. The mechanistic and structural studies described herein will help in the rational design of efficient inhibitors for human EGFR and ErbB2 that will be useful in treating breast cancer.

BODY

Aim 1. How do Argos and Kekkon bind to the *Drosophila* EGF receptor (dEGFR) ? (Months 1-10)

To determine how Argos and Kekkon inhibit the *Drosophila* EGF receptor (dEGFR) an efficient system for the production and purification of the extracellular domains of Argos, Kekkon, dEGFR, and a dEGFR agonist (Spitz) first needed to be developed. I have subcloned the entire Argos cDNA (with a C-terminal 6x histidine tag) behind the metallothionein promoter of a pMT vector, for Cu²⁺ inducible expression in *Drosophila* Schneider 2 cells. I have established a stable cell line that expresses histidine-tagged Argos, and can now produce milligram quantities of protein. The protein is first purified by utilizing the nickel binding (6x histidine) tag using a Ni-NTA matrix for immobilized-metal affinity chromatography (IMAC). This procedure is then followed by a size exclusion gel filtration chromatography purification step that yields protein of greater than 90% purity. Similar production strategies have been employed for the successful production of the extracellular or secreted forms of the *Drosophila* EGF receptor, Kekkon (1 and 2) and Spitz. Stable cell lines of each protein have been developed and milligram quantities of each protein can be purified(14).

Using surface plasmon resonance (SPI-BIAcore) technology I have investigated the binding of secreted Spitz and Argos to a secreted extracellular form of dEGFR (sdEGFR). The ligand (either Spitz or Argos) was immobilized on a CM5 biosensor chip using standard amine-coupling chemistry, and purified sdEGFR as analyte was passed over this surface at a series of different concentrations to generate an equilibrium binding curve. This approach has been previously used extensively in the Lemmon laboratory to analyze binding of mammalian ErbB ligands to their receptor extracellular domains. Our results show that Spitz binds to sdEGFR with a K_d of ~160nM (Figure 1c, 14), which is very similar to the reported K_d's of human EGF to the human EGF receptor ~130nM. However, we could not detect any binding of Argos to sdEGFR (Figure 1a, 14). To our

surprise, and contrary to popular belief(15-18) Spitz bound to the same immobilized Argos chip with an affinity nearly 10 times stronger than the Spitz-sdEGFR interaction (Figure 1b, 14). Reversing the experiment, with immobilized sdEGFR as the prepared surface, we again were able to see binding of Spitz to sdEGFR but Argos failed to give any response to the receptor chip.

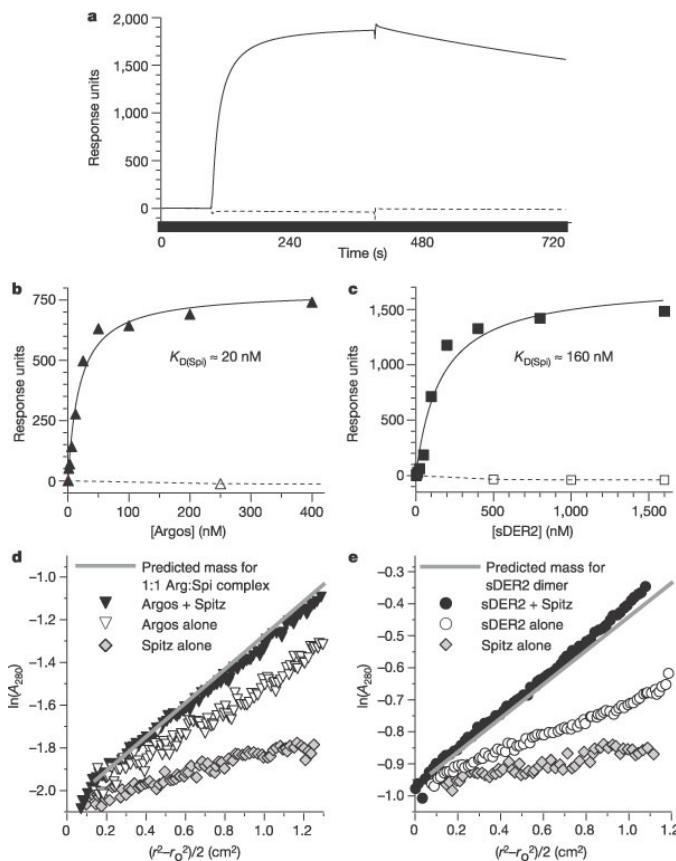


Figure 1 Quantitative analysis of Spitz/Argos interactions. **a**, SPR sensorgrams show that Spitz (at 1 μ M, solid line), but not dEGFR (sDER) (at 1 μ M, dotted line) binds immobilized Argos. **b**, Representative binding curves for interaction of soluble Argos with immobilized Spitz (filled triangles) and immobilized sDER (open triangles). **c**, sDER2 binds immobilized Spitz (filled squares) but not immobilized Argos (open squares). **d**, In analytical ultracentrifugation, Argos (open triangles) sediments at 48.8 ± 4.3 kDa (47.6 kDa expected without glycosylation), whereas Spitz (diamonds) sediments as a 16.2 ± 1.7 kDa species (11.8 kDa expected without glycosylation). Equimolar Argos/Spitz mixtures fit best to a single ~ 59 kDa species (filled triangles). The grey straight line represents expected results for a 1:1 Argos:Spitz complex. **e**, sDER (open circles) sediments as a 101.8 ± 0.6 kDa species, which approximately doubles upon adding a 1.2-fold excess of Spitz (filled circles); Spitz alone (diamonds) sediments as in **d**. The grey line represents expected results for an sDER dimer. (14)

Aim 2. Do the inhibitors compete with agonists for binding to the receptor ? (Months6-10)

Our initial surface plasmon resonance experiments revealed that Argos will not compete with Spitz for binding to the receptor. Because we determined that both Argos and sdEGFR bind to Spitz we hypothesized that they may compete for the same binding site on Spitz. Therefore our competition studies focused on agonist binding rather than receptor binding. A surface of sdEGFR was prepared and an analyte of 250nM Spitz was passed over this surface and gave a response of ~ 600 RU. Then an increasing amount of Argos was added to the analyte while the Spitz concentration was held constant at 250nM. The addition of increasing amounts of Argos progressively reduced the signal response until ultimately no response was detected when the ratio of Argos to Spitz was ~ 1.1 (Figure 2a, 14). Since the response units are directly related to the mass bound to the surface, the only explanation for the given result is that Argos sequesters Spitz away and

prevents it from interacting with the sEGFR surface. The finding that this interaction is abolished at equimolar concentrations of agonist and inhibitor (when performed at greater than 10 times Kd) demonstrate that the stoichiometry of the interaction in solution is 1:1.

To examine the ability of Argos to act as a ligand sink in a cellular context we used a *Drosophila* S2 cell line that expresses dEGFR. Addition of Spitz at concentrations of 10nM, 50nM, or 100nM induced robust activation of the receptor as detected by an antibody that recognizes dEGFR tyrosine autophosphorylation. Then, consistent with the biosensor analysis, increasing amounts of Argos progressively inhibited the autoactivation of the receptor (Figure 2b, 14). The finding that a 5 fold or greater amount of Argos is needed for complete inhibition in the cellular context suggests that Spitz binds tighter to the cell surface than in the biosensor analysis (this has been shown to be true for the human system).

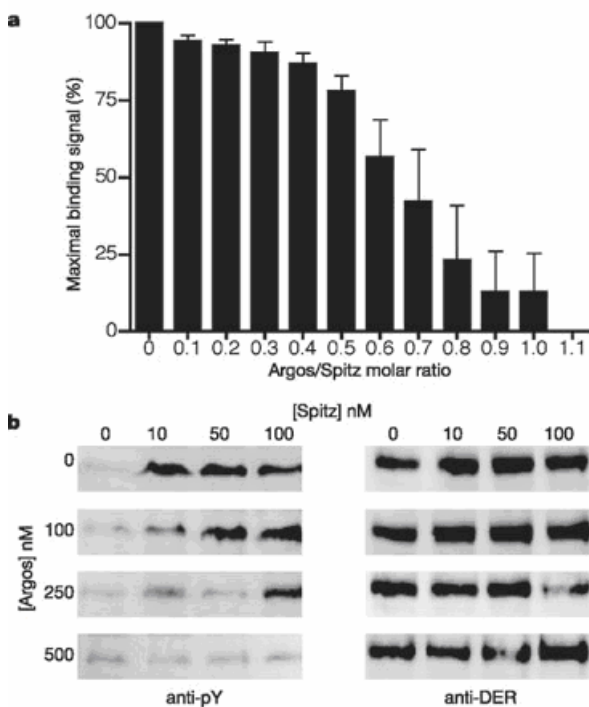


Figure 2 Argos sequesters Spitz away from dEGFR (DER). **a**, Binding to immobilized sDER was measured for samples containing 250 nM Spitz and the indicated mole fraction of Argos. Binding is abolished when Argos is in excess. Mean \pm s.d. ($n \geq 3$) is shown. **b**, Western blot showing robust Spitz-induced tyrosine phosphorylation of DER expressed in S2 cells and the effect of adding different concentrations of Argos, using an anti-phosphotyrosine antibody (anti-pY, left panels). Western blot showing DER protein levels using an anti-DER antibody (right panels). (14)

Our further research on the mechanism of dEGFR binding of Kekkon has confirmed earlier reports that Kekkon interacts directly with the receptor. This interaction seems to be confined to domain 5 of dEGFR, a domain that is not found in the human receptor. Thus, this interaction may not effect ligand binding by the receptor, which occurs at domain 1 and 3, but may effect dimerization and activation of the receptor. Future studies will address this possibility more directly.

Aim 3. Do Argos and Kekkon directly inhibit dimerization of dEGFR ? (Months 8-12)

Analytical sedimentation equilibrium ultracentrifugation studies show that sdEGFR runs at its monomeric size. Upon addition of a 1.2 fold excess of Spitz this size approximately doubles, revealing that the expected 1:1 interaction of Spitz to sdEGFR causes dimerization of the extracellular portion of the receptor (Figure 1e, 14). Spitz alone runs at approximately 16kD, slightly above the predicted 12kD but consistent with several predicted glycosylation sites. Argos sediments very near its predicted size of 48kD. An equimolar mixture of Argos and Spitz sediments at nearly 60kD, further demonstrating the 1:1 stoichiometry of the complex (Figure 1d, 14).

As stated above, Kekkón binding of domain 5 of dEGFR likely suggests its mechanism of action is to prevent the formation of an active dimer of the receptor rather than to prevent ligand binding. Future studies will address this question specifically, however it is hampered in part by the very low affinity of sKekkón for sdEGFR. The low solution affinity is likely readily overcome *in vivo* when these molecules are restricted to the 2 dimensional membrane.

Aim 4. Do the inhibitors prevent 'unlocking' of the dEGFR autoinhibited form ? (Months 12-24)

Mutants of sdEGFR that should prevent dimerization and/or tethering (based upon homology to the human receptors) have been cloned and expressed in the same manner as wild type sdEGFR. When mutations are made in human EGFR to prevent this autoinhibited form the K_d of ligand binding, as measured by biosensor, goes down, demonstrating an increased affinity. However when the same mutations are made in dEGFR the ligand affinity stays the same or is reduced. This difference between the human and fly system was surprising, so we then looked at a low resolution structure of EGFR, ErbB2 and dEGFR by SAXs (small angle x-ray scattering). Briefly, this technique allows us to view a molecular envelope for a given structure and to get a measure of the maximal dimension of the molecule. This experiment allowed us to compare dEGFR with the autoinhibited EGFR and the fully untethered ErbB2. In these experiments dEGFR resembles the “open” ErbB2 and cannot fit into the “tethered” envelope of EGFR. Thus, it seems that dEGFR does not undergo the same autoinhibition that is found in the human system and Kekkón therefore could not help to stabilize such an inhibition and prevent ‘unlocking’ of the receptor.

Aim 5. Do the putative human Argos and Kekkón homologs that I have inhibit human erbB receptors ? (Months 10-32)

To further investigate if the putative human Argos and Kekkón homologs that I have found inhibit human ErbB receptors, I have first generated stable cell lines that express IGFBP-rP1 and the extracellular region of NAG-L in a similar manner to the production of Argos and Kekkón. Both proteins failed to bind human EGFR as well as the human ligands EGF and Neuregulin when measured using biosensor.

**Aim 6. What is the structural basis for dEGFR inhibition by Argos and Kekkon ?
(Months 6-36)**

The discovery of Argos as a ligand 'sink' has prompted us to focus on the structure this novel interaction. The wild type Argos protein is highly differentially O- and N- link glycosylated and prone to degradation making it unsuitable for crystallography trials. Attempts have been made to produce non glycosylated versions of Argos that are much less prone to degradation. We now can produce a very stable Argos that is non glycosylated and that retains wild type affinity to Spitz. This construct Argos217 is just 217 amino acids of the original 444 amino acid wild type Argos. Argos217 can be produced from S2 cells, as well as from Sf9, cells with a final yield of purified protein at nearly 1mg/L of culture. The protein is purified by IMAC followed by ion exchange and then size exclusion chromatography to a purity >95%. After an initial crystal screen, an optimized condition of 0.1M Actetate pH 5.4, 15% PEG 6000, 0.2M Ammonium Sulfate was found to produce diffraction quality crystals. Diffraction was obtained at ALS (Berkeley) and was 97% complete to 2.6 Å. The crystal space group is C2 with a unit cell (A:112, B:64, C:72, α :90, β :101.3, γ :90). Because Argos represents a novel structure a derivative set must be obtained for the solution. Future work will focus on obtaining diffraction quality crystals from heavy atom soaks as well as Seleno-methionine derivitized protein.

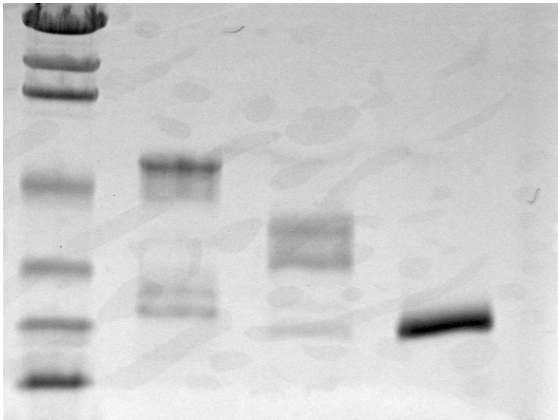


Figure 3 Lane 1 is molecular weight marker; Lane 2 is wild-type Argos; Lane 3 is Argos with a linker of 5 amino acids replacing the 120 amino acids of Drosophila Argos; Lane 4 is further reduced to Argos217 which is clearly less glycosylated and degraded. All forms produced from Schneider 2 cells. Aprx 5ug of each run on a 12.5% Coomassie gel.

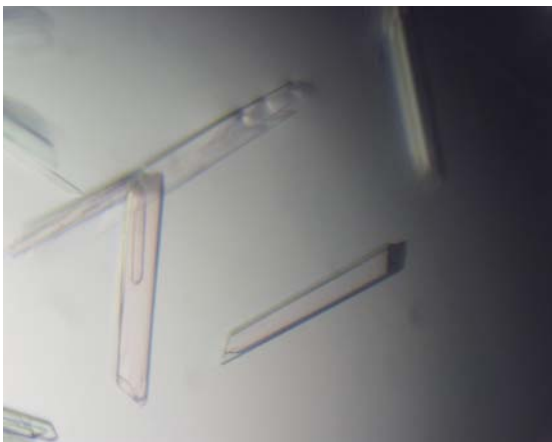


Figure 4 Crystals of Sf9 purified Argos217 obtained from the hanging drop method at 21°C in a mother liquor of 0.1M Actetate pH 5.4, 15% PEG 6000, 0.2M Ammonium Sulfate. The drop was set up 1:1 protein to mother liquor with Argos217 at 6.3mg/mL

Production of a crystal quality ligand (Spitz or Vein) for Argos is challenged by poor yields and heavy O- and N- linked glycosylation . Crystal screens of an Argos:Spitz complex have been attempted with fully glycosylated secreted Spitz with no initial hits. Future work will focus on attempting to produce a non-glycosylated Spitz EGF domain to further screen conditions for complex crystal formation.

To get a greater understanding for the recognition requirements of Argos for EGF ligands we determined its affinity to another dEGFR ligand Vein. Vein has the greatest sequence divergence from Spitz of the 4 dEGFR ligands and is thought to be more neuregulin like because of the presence of an Ig domain just preceding the EGF domain at the extreme C-termini of the protein. Using surface plasmon resonance we examined the ability of the Vein EGF domain to bind dEGFR as well as Argos. We discovered that Vein seems to selectively retain high affinity to Argos. The K_d of the interaction is nearly as strong as Spitz (60nM as compared to 20nM), while the affinity is greatly reduced for the receptor (>10uM as compared to 160nM). From this finding, and by comparing the sequences of the EGF domain of Vein and Spitz, only several residues remain identical outside of the consensus EGF fold. We hypothesize that some of these residues may dictate the high affinity interaction between Argos and the EGF domain. Therefore, we have mutated these residues individually to Alanine to investigate their effect on Argos binding. These results should shed light on the interaction of Argos and EGF domains in general and along with a structure may lead to the ability to produce EGF binding proteins specific to other EGF domains, namely those involved in human breast cancer (EGF, TGF- α).

Key Research Accomplishments

- Ability to produce and purify milligram quantities of sdEGFR, Argos, Spitz and Kekkon.
- Demonstration that the *Drosophila* EGF (Spitz) binds to sdEGFR with a K_d comparable to that of the human system. Also, we show that purified Spitz can lead to autophosphorylation of dEGFR expressed in S2 cells.
- Discovery that Argos represents a novel ligand sequestration mechanism for inhibiting EGFR signalling.
- Demonstration that Kekkon acts by binding specifically to domain 5 of dEGFR. And that Kekkon can not stabilize an autoinhibited form of dEGFR that resembles that of human EGFR.
- Discovery that a second dEGFR ligand (Vein) binds with high affinity to Argos.
- Production of crystal quality Argos and diffraction data of a native set 97% complete to 2.6Å.

Reportable Outcomes

- Original Research Publication
Klein DE, Nappi VM, Reeves GT, Shvartsman SY, & Lemmon MA
Argos inhibits EGFR signalling by ligand sequestration.
Nature **430**, 1040-1044; 26 August (2004).
- Original Research Publication
Reeves GT, Kalifa R, Klein DE, Lemmon MA, Shvartsman SY.
Computational analysis of EGFR inhibition by Argos.
Developmental Biology **284**, 523-535; August 15 (2005).
- Bayard T. Storey Award (2005) for excellence in research.
Center of Research on Reproduction and Women's Health. University of
Pennsylvania School of Medicine
- Dr. Roy G. Williams Prize (2005) for meritorious research in the medical sciences.
University of Pennsylvania School of Medicine.
- Poster Prize 3rd Place (2006) University of Pennsylvania School of Medicine,
Department of Biochemistry and Biophysics, Departmental Retreat

CONCLUSIONS

Our studies have uncovered a novel mechanism of EGF receptor inhibition. In our examination into the mechanism of inhibition of dEGFR by Argos we unexpectedly discovered that Argos functions as a ligand sink rather than a competitive antagonist or inverse agonist of the receptor as previously thought(14). This type of inhibition is unprecedented in the EGF family but is consistent with other known extracellular receptor inhibitors (e.g. IGF-Binding Proteins, Noggin, Chordin, Cerberus)(19). Future studies will address the specificity of inhibition of Argos for the remaining 3 ligands in *Drosophila*, and whether a mammalian Argos like molecule exists. Since discovering this novel mechanism of inhibition, a great deal of effort has been made to produce crystal quality protein to ultimately get a physical understanding of this sequestering event. We now can produce crystal grade protein and have preliminary diffraction data. Further studies will focus on obtaining derivative data sets, which are necessary to solve the structure of this novel protein. Finally, our demonstration that ligand sequestration can efficiently regulate the EGFR axis may provide a basis for the development of future therapies that neutralize erbB receptor agonists when such overexpression contributes to oncologic processes.

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